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YAC transgenesis: a study of conditions to protect YAC DNA from breakage and a protocol for transfection

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Abstract

Yeast artificial chromosomes (YACs) are providing a great boon to transgene technology by allowing the easy mutagenesis and study of very large DNAs. The large insert sizes of these vectors permit more accurate analysis of the regulation of transgene expression than smaller, more artificially assembled constructs. Transfection of mammalian cells by YACs can be accomplished by a number of methods; the most prevalent, using gel-purified DNA, is dependent upon compaction by salts to protect the large YAC DNA from breakage. We show that the common reliance on NaCl to compact YAC DNA sufficiently to protect it from breakage is not well-founded. Even the use of mixtures of polyamines and NaCl allows substantial damage to purified YACs. The use of polyamines alone in low salt buffers to compact YAC DNA provides the best protection from breakage and allows very effective transfection of murine embryonic stem (ES) cells. We provide a detailed method for ES cell transfection by YACs utilizing the DOTAP lipofection reagent that optimizes transfection efficiency and recovery of intact YACs. © 1998 Elsevier Science B.V.

Keywords: Yeast artificial chromosome; Transgene; Transfection; Polyamine; Spermine; DNA condensation

1. Introduction

Yeast artificial chromosomes (YACs) are being increasingly used to transfect tissue culture cells and to produce transgenic mice. The large insert sizes (100 kb–2 Mb) of these vectors allow more accurate reproduction and analysis of mammalian gene expression than smaller and more artificially assembled constructs. YACs can also be used for in vivo screening of large chromosomal regions for genetic elements such as origins of replication and methylation determinants [1]. Compared to other vectors, YACs have the added advantage that their DNA inserts can be easily altered due to the very high levels of homologous recombination found in the yeast host. It is possible that YAC transfection will become even more important in the near future as it is applied to complementational cloning of disease genes such as tumor suppressors, and to genes whose disease phenotypes are associated with increased gene dosage, such as in Down's Syndrome [2,3].

Since YACs are large they pose some technical problems in terms of obtaining adequate quantities of DNA for transfection and keeping the DNA from being sheared during its preparation and use. The first attempts to make cells transgenic with YACs solved

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these problems by directly fusing YAC-bearing yeast spheroplasts to mammalian cells [4–7]. However, this method introduced much of the yeast genome into the recipient cells along with the YACs, which in some cases might have had an unwanted impact on the cell phenotype [6]. Therefore, despite the convenience of the technique and its proven ability to introduce intact, functioning transgenes which can be transmitted through the mouse germline [7], many investigators sought a means to transfect more purified YACs.

A number of different schemes have been used to protect gel-purified YACs from breakage during transfection into tissue culture cells or microinjection into the pronuclei of fertilized oocytes. These approaches all attempt to reduce the radius of gyration of large linear DNAs by compacting them in various salts. The salts used for protecting YACs can be broken down into three classes: (1) polyamines such as spermidine^{+3} and spermine^{+4}, (2) Na^+, and (3) combinations of polyamines and Na^+.

We have examined the use of polyamines and sodium to protect YACs from breakage and to obtain an optimized yield of transfected ES cells containing intact YACs. Our data show that the reliance on Na^+, either alone or in combination with polyamines, will not provide protection of YACs comparable to that from polyamines alone. We also show that the presence of sodium will antagonize the protective effects of the polyamines on YAC DNA. Nonetheless, we illustrate that the addition of sodium to previously protected and concentrated YAC DNA may be beneficial for lipofection. We also discuss several aspects of the transfection protocol which can have a substantial impact on the successful production of transgenic YACs. Among the salient technical findings, we show that, in contrast to prevailing claims [8], not all sera are compatible with lipofection using *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP); fetal bovine serum, in particular, can be highly inhibitory to lipofection.

2. Materials and methods

2.1. Reagents

MIB: 10 mM Tris pH 7.5, 250 μM disodium EDTA pH 8.0. 50 mM spermine (tetrahydrochloride; Sigma S-1141): 0.2 μm filtered and stored as aliquots at −20°C. SeaPlaque GTG low melting point agarose (FMC): an occasional lot showed some ES cell toxicity at volumes above 300–400 μl of digested gel; most showed no effect up to at least 700 μl (e.g. lot 729295). β-Agarase (New England Biolabs). Centricon-100 concentrator (Amicon, Beverly, MA). DOTAP (Boehringer-Mannheim): lot 14854800 is very effective; lot 14851200 is not. ET: 50 mM EDTA pH 8.0, 10 mM Tris pH 7.5. ESP: *N*-lauroyl sarcosine (sarkosyl), 10 mM Tris pH 7.5, 0.5 M EDTA pH 8.0; 10 mg Proteinase K (2 mg/ml final) was added to each 5 ml ESP just before use. LET: 0.5 M EDTA pH 8.0, 10 mM Tris pH 7.5; 2.2 ml β-mercaptoethanol (β-ME) was added to each 28 ml LET immediately before use. Zymolase-100 T (Seikagaku, Bethesda, 1-800-237-4512): 2–7.4 mg/ml in 10 mM $NaPO_4$ pH 7.5, 50% glycerol; stored at −20°C. 100 mM PMSF: 174 mg PMSF was dissolved in 10 ml isopropanol and stored in 1 ml aliquots at −20°C. 1 M Hepes pH 7.4 (GIBCO-BRL, Life Sciences). Wide bore pipet tips (USA/Scientific Plastics, Ocala, FL, 1011-8406). 2.0 ml tubes (Sarstedt, 72-693-005). Pure polystyrene tubes (Falcon 2037). ES Medium: 15% fetal bovine serum (FBS; Hyclone lot 11152557), L-glutamine, penicillin–streptomycin, pyruvate, and non-essential amino acids (GIBCO-BRL; 1/100 dilutions), β-mercaptoethanol (3.6 μl/500 ml), LIF (from CHO cell line 8/24 720 LIF-D.1, Genetics Institute). FBS$^-$ Medium: ES medium without FBS, pyruvate, non-essential amino acids, and LIF.

2.2. Yeast chromosomal plugs

YAC-bearing yeast from a selective plate were grown overnight (O/N) in 10 ml YPD liquid medium and then used to inoculate 500 ml YPD in a 1 l flask. After shaking at 30°C up to 24 h, the cells were at least 1×10^8/ml for AB1380 strains. 1% low melting point agarose (SeaPlaque GTG) in 125 mM EDTA was melted and then cooled to 54°C. 500 ml of cells were pelleted ($3700 \times g$, 5 min, e.g. 6000 rpm, GSA rotor), resuspended in 10 ml ET, transferred to 50 ml polypropylene tubes, and pelleted again ($1200 \times g$, 7 min, e.g. 2600 rpm IEC rotor). 8 ml of 1% agarose were transferred to 42°C. The pellet was resuspended in 350 μl ET by mixing with the tip of a 1 ml pipet.

A 1/100 dilution of 5 μl was counted; the cells were diluted with ET to achieve 1.0×10^{10}/ml (or less – see Section 4). 160 μl of 2 mg/ml of Zymolase 100 T were added to each 8 ml of agarose at 42°C and mixed by inversion. 2.5 ml of yeast solution was transferred to a dish (e.g. 6 cm tissue culture) propped at an angle. An equal volume of the Zymolase/agarose was added to the dish and mixed rapidly with the cells by drawing the solution into a 5 cc syringe and expelling once. The cell-agarose mixture was again drawn into the syringe, capped, and plunged into ice. The procedure was repeated for all of the cell volume. Hardened plugs were poured into a 15 ml round bottom tube (e.g. Falcon 2059) and cored with a sterile Pasteur pipet in order to provide more rapid diffusion of buffer. The plugs were covered with 6–7 ml of LET + β-ME (28 ml LET + 2.2 ml β-ME). The tubes were sealed with Parafilm and placed at 37°C for 8–16 h, after which the supernatant was poured off and the plugs rinsed with 5 ml of ET. Upon addition of 6 ml of Proteinase K (2 mg/ml) in ESP, each plug was incubated at 50–54°C for 16–24 h. A second Proteinase K treatment (approximately 8 h the next day) was performed to increase the shelf life of the thick plugs used here. After the final Proteinase K treatment, the plugs were rinsed with ET, and then soaked for 2–3 h, at room temperature (RT) in 8 ml of ET + 80 μl 17.4 mg/ml PMSF in isopropanol. The plugs were then soaked twice more in 8 ml ET for 3 h, RT or O/N, 4°C followed by a final soak in 5 ml ET + 1/100 volume of 10 mg/ml RNase A at RT. For short term storage (at least several months) the YACs were kept at 4°C in ET.

2.3. Isolation and concentration of YAC DNA

YAC plugs were loaded onto a 250 ml CHEF gel (1% SeaPlaque GTG low melting point agarose in 0.5 × TBE, 2 l) with a common 18.5 cm central slot flanked by two marker lanes. Usually, almost 4 g of YAC plug was loaded, corresponding to approximately 250 ml of original culture. One-half of the YAC isolated from this gel was used for a single transformation of 1.5×10^7 ES cells. Conditions used for good separation of β-globin YACs in the 200–300 kb range were: 15–30 s switch, 40 h, 6 V/cm (200 V, CHEF-DRII), 14°C. Under these conditions a 250 kb YAC was generally well enough separated from the nearest yeast chromosome (I) that yeast genomic DNA contamination was minimized; however, the degree to which YAC DNA will be devoid of yeast genomic DNA will depend on the overlap between the YAC and neighboring yeast chromosomes on the CHEF gel (see Section 4). Marker slices from the edges of the common well through the flanking marker lanes were stained in ethidium bromide (0.5 μg/ml) as was a slice from the center of the gel. The marker chromosomes were visualized with UV light and cuts placed above and below the band of interest. The CHEF gel was reassembled and unstained gel pieces corresponding to the marker slices were excised. The gel slices containing the YAC were weighed, diced into small blocks, and added to rinsed 100 ml glass bottles. A typical slice from one half of a 250 ml CHEF gel weighed from 7–10 g. Slices were stored at 4°C in running buffer without negative effect on the DNA for at least one week.

The timing of ES cell growth and YAC preparation were scheduled as follows (Fig. 5): when ES cells on a 10 cm plate were two days prior to confluence (usually 1–2 days after thawing), the first overnight soak of the YAC agarose blocks was performed. The blocks were soaked in 10 volumes MIB/100 μM spermine. The soaks were continued the next day for 3–8 h, RT, and then overnight at 4°C (for a final dilution of 1000 ×). The agarose was melted by removing the buffer and submerging the bottle in a 70°C water bath for 15 min (swirling once gently after 8–10 min). The bottle with melted gel was then equilibrated in a 52°C water bath for 10 min. (Additional buffers such as Bis–Tris were found not to be necessary for complete β-agarase activity.) 2 units/100 μl gel of fresh β-agarase equilibrated at RT for 1–2 min was added and the liquid gel then mixed by swirling gently 10 ×; incubation at 52°C proceeded for approximately 2 h. The bottle was then equilibrated to room temperature and all of the liquid gel was transferred to a 15 ml conical tube with a 1 ml pipet and spun at 200 × *g* (e.g. 1000 rpm, Beckman TJ-6, swinging bucket rotor; equivalent to approximately 1500 rpm in a microfuge) for 15 min. If the digestion was complete, no agarose pellet was seen. Often, the YACs were concentrated in a loose ''pellet'', i.e. roughly in the bottom 0.3 ml of the

cleared solution. The bottom 0.3 ml of the cleared supernatant was made 200 mM in NaCl and incubated O/N at RT. The agarose supernatant was stored overnight at 4°C. On the same day that fractionation of the digested agarose was performed, a 10 cm plate with confluent ES cells was split into two 10 cm feeder plates for transfection the following day.

On the day of transfection, the digested agarose supernatant containing YAC DNA was transferred to Centricon-100 centrifugal concentration units (Amicon) and spun at $200 \times g$ until the fluid was concentrated to approximately 200–300 μl (from < 15 min to > 1 h depending on the actual extent of digestion). The concentration step was followed by a gentle 50 μl rinse of the filter with MIB/SP flowthrough or the YAC solution itself (pipetting 3 × with a wide-bore pipet). The concentration of YAC DNA in the fractions containing the aggregated ''pellet'' and concentrated supernatant DNAs were measured, e.g. by running 10 μl of each on an agarose 1/2 × TBE minigel versus λDNA standards of 20, 8, 4, 1.6, and 0.8 ng (which had been deconcatenated by heating for 5 min at 65°C). Once the xylene cyanol dye had run below the DNA, a Polaroid photograph was taken and scanned into a graphics program (e.g. the NIH Image program which was downloaded from http://rsb.info.nih.gov/nih–image) for rapid quantitation. If the YAC DNA did not adequately migrate into the agarose gel, it was possible to measure the concentration of the YACs using ethidium bromide spots. The initial YAC concentration was usually approximately 0.1–0.2 ng/μl. The final concentration of the YACs was at least 2.5 ng/μl and never more than 4 ng/μl, since YAC precipitation which is difficult to reverse occurred above this concentration. 300 μl or more of 4 ng/μl 250 kb β-globin YAC included variable amounts of the similarly sized Chromosome I of yeast. On some occasions, YAC DNA of 4 ng/μl or less was stored at 4°C for at least one week without degradation or losses, but usually the YACs were used immediately after concentration to reduce the risk of aggregation. A 1% CHEF gel was used to confirm the concentration of the YACs, and their intactness if NaCl was present during purification (see Section 3); 1/10th of the final volume of concentrated YAC DNA was run in one lane against appropriate markers and concentration standards.

2.4. YAC transfection of ES cells

Approximately 6×10^7 ES cells were usually available after having been split 1:2 the day prior to transfection; these cells were sufficient for four transfections. If analysis of the transgene was to occur in chimeric animals, the ES cell GPI allele was distinguishable from that of the host blastocysts, e.g. CCE ES cells (GPI-c) and MF1 blastocysts (GPI-a). The ES cells were trypsinized and resuspended in 100 μl residual medium. The cells were washed twice with 10 ml FCS^- medium, resuspended in 4 ml FCS^- medium, and counted. The volume was adjusted with FCS^- medium so that the cell concentration was approximately 5×10^6/ml (usually 12 ml final). The cells were stored at 4°C.

Up to three control transfections were often added in parallel with that employing YAC DNA compacted with 100 μM spermine: (1) A sample of unconcentrated YAC DNA protected in 100 μM spermine, (2) 20 ng of a gel-purified, 5 kb DNA fragment containing PGKneo, and (3) A YAC sample prepared in 100 μM SP + 200 mM NaCl as a further backup against unexpected DNA losses (Section 3, Fig. 5). The DNAs were added to 50 μl of 20 mM HEPES pH 7.4, and 30 μl (30 μg) fresh DOTAP lipofection reagent was added to 100 μl 20 mM HEPES pH 7.4. The DNA and DOTAP were mixed by transferring them with a 1 ml pipet to a pure polystyrene tube; after incubation for 30–45 min at RT, the cells were gently pipetted onto the DOTAP/DNA mix. The DNA-cell mixture was incubated 30 min at RT, and rocked extremely gently once every 7–8 min when the cells started to settle. (It was at this stage, when diluted out of the spermine, that the DNA appeared to be most susceptible to breakage). The transformation was then very gently poured onto a 10 cm feeder plate which had been washed once with 10 ml PBS and which contained 3 ml FCS^- medium + 2 × LIF at the concentration optimized for the ES cell line in use. After 12 h of incubation at 37°C, the FCS^- medium was changed to 10 ml ES medium.

Approximately 1.5–2 days after the transfection when the colonies were near confluence, G418 was added to 200 μg/ml. The first colonies to pick were easily visible to the naked eye 7 days after addition of G418; they were picked to 96-well feeder plates. Another round of colonies were often picked 10 days

after addition of G418 and up to 13–15 days. Half of the confluent 96-well ES cell growth was transferred to a gelatinized 96-well plate, and the other half of the cells were frozen at −70°C [9]. When the cells in the gelatinized 96-well plate were confluent, genomic DNA was made and the YAC scanned for retention of markers by PCR. Those YACs which had retained all PCR markers were thawed and expanded to make CHEF plugs and genomic DNA. Pulsed-field gel analysis allowed determination of which ES clones had 1–2 copies of intact YAC without any broken fragments. The copy number was often confirmed by Southern blotting; the same blots were then used to fingerprint the clones by hybridization of a Blur8 Alu probe [10] at 65°C in buffer containing 5% SDS [11]. Genomic DNA was run out as far as possible (e.g. 19–20 cm) to diffuse the background without affecting the specific signals of the Alu fingerprints.

3. Results

3.1. Polyamines provide the best protection against YAC breakage

Parallel YAC samples, differing only in the salts used to protect the YAC DNA before release from agarose slices, were prepared by the protocol described (Section 2). Spermine (100 μM), NaCl (100 mM), a mixture of polyamines (30 μM spermine and 70 μM spermidine), or the same polyamines plus

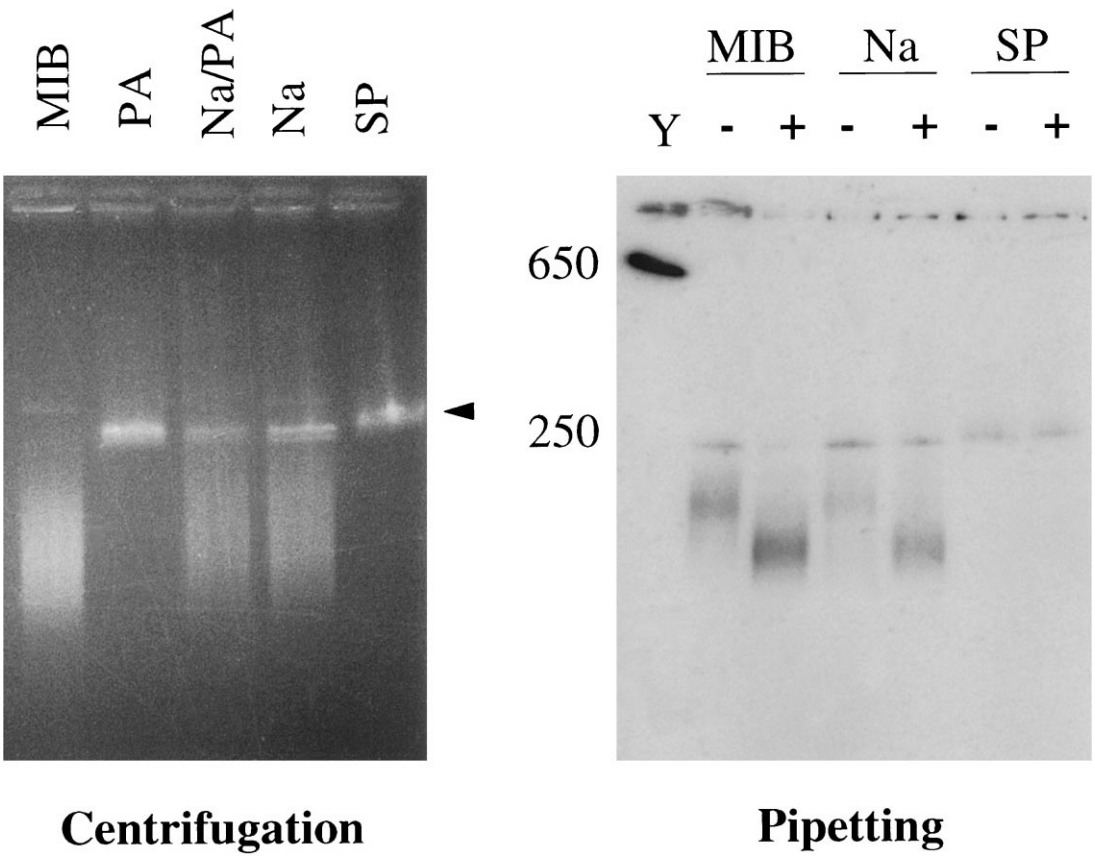


Fig. 1. NaCl does not provide DNA protection comparable to that of polymines. Various salts (PA: 70 μM spermidine + 30 μM spermine; Na: 100 mM NaCl; Na/PA: PA + Na; SP: 100 μM spermine) were compared under equivalent conditions (in a microinjection buffer, MIB) for their effect on the breakage of a 250 kb YAC. The intact YAC band is indicated by an arrow (left gel) or ''250'' (right gel). Left gel: An ethidium bromide stained CHEF gel showing YAC DNA which was centrifuged and concentrated for 1 h., 200 × *g*, in a Centricon 100 unit. Right gel: A Southern blot of a CHEF gel hybridized to a yeast LYS probe showing YAC DNA ± pipeting 15 × through a normal bore 200 μl pipet tip. ''Y'' shows a 650 kb yeast chromosome hybridizing to a probe from the *LYS2* gene.

100 mM NaCl [12] were compared for their ability to resist YAC breakage by centrifugation (Fig. 1, Left). After concentrating the YACs by spinning in a microconcentrator (Amicon), we found that only the spermine and polyamine soaked YACs remained grossly undamaged. Not only were the YACs which had been soaked in 100 mM NaCl partially broken, but a similar degree of breakage was seen when the polyamines were combined with 100 mM NaCl. In a related experiment, YACs soaked in spermine or sodium were pipetted fifteen times vigorously through a normal bore 200 μl pipet tip (Fig. 1, Right). Again, in contrast to the results of Gnirke et al. [13], we found that 100 mM NaCl alone did not protect the DNA from being sheared, and did not provide protection comparable to that seen with 100 μM spermine.

To further examine the protection afforded by the various salt treatments, we prepared parallel YAC samples either in polyamines or spermine (Section 2), or in sodium-containing salts exactly as described previously [12,13]. Completely intact YACs were obtained by all of these methods (Fig. 2 shows each of the YAC samples after storage at 4°C for 3 days, by which time some breakage had occurred). We then attempted to simulate the shear forces which YACs might experience upon passage through a microinjection needle. In order to recover sufficient DNA for gel analysis, YAC samples were pushed through pulled glass needles of a bore larger than that ordinarily used for microinjection (12 μm vs. 0.5 μm). In this case, once again, the polyamine soaked YACs remained intact while the sodium soaked YACs did

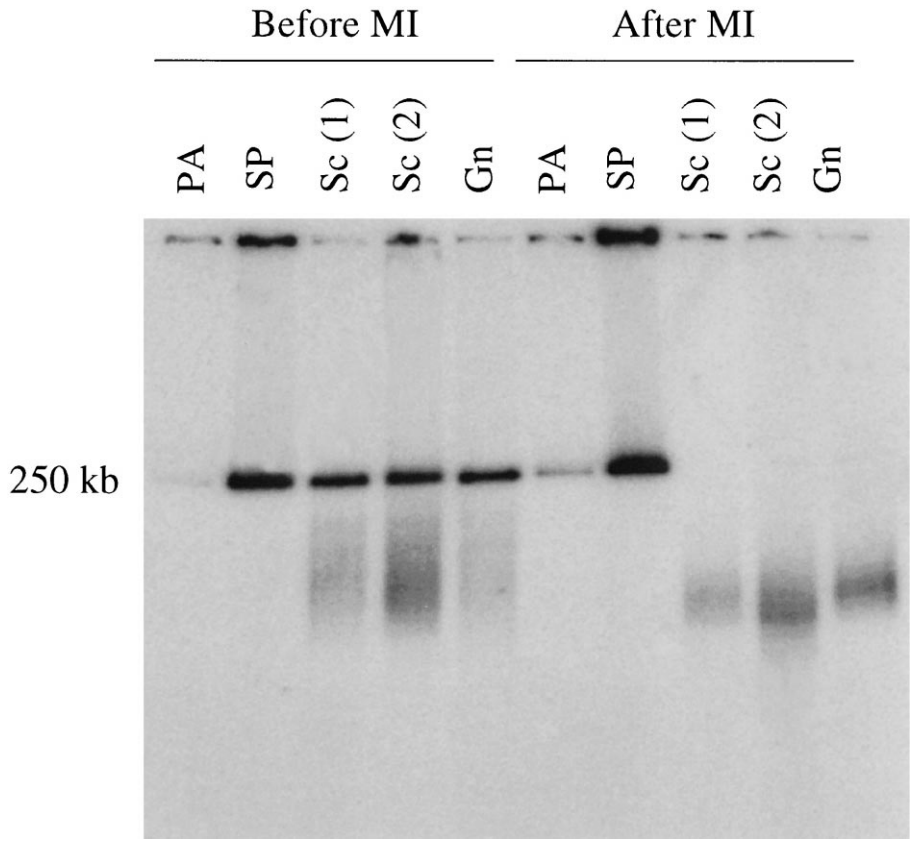


Fig. 2. YAC DNA ''Microinjection'' Test. YAC DNA was prepared in ''Sc'' buffer containing 100 mM NaCl, 70 μM sperminine, and 30 μM spermine [12] or in ''Gn'' buffer containing 100 mM NaCl [13] exactly as described. ''PA'' and ''SP'' are YAC samples prepared in 70 μM spermidine/30 μM spermine or 100 μM spermine as described in the text. ''Before MI'' shows the samples after 3 days at 4°C, immediately before the experiment. Each sample was passeed once through a glass injection needle with a 12 μ bore and then loaded on a CHEF gel, which was then blotted and hybridized to an ε-globin probe. The ''Sc'' samples are shown in duplicate.

not (Fig. 2). Although the shear stress we used is not exactly the same as that found in a microinjection pipet used to introduce DNA into pronuclei, our data suggest that a higher yield of intact YACs may be obtained during microinjection from pure polyamine rather than buffers containing 100 mM NaCl.

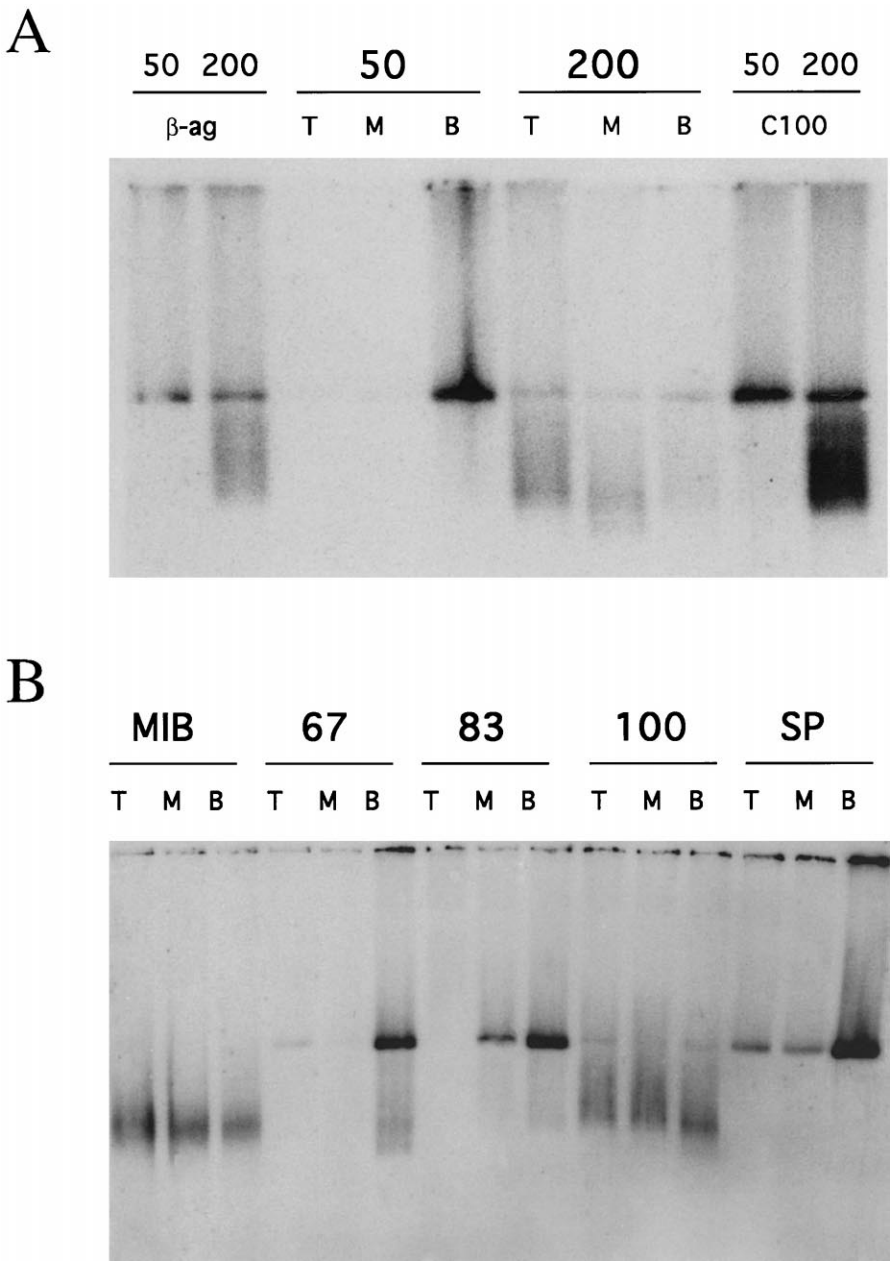


Fig. 3. Titration of the effect of NaCl on YAC aggregation and resistance to breakage. YAC DNA was prepared in the presence of 100 μM spermine and 50 mM or 200 mM NaCl. The DNA was centrifuged at 200 × *g* for 15 min at room temperature and divided into three fractions. ''T'', ''M'', and ''B'' (top, middle, and bottom). Aliquots from each fraction were analyzed by CHEF electrophoresis, blotting, and hydridization with a human *ε*-globin probe. (A) ''*β*-ag'' shows the samples after *β*-agarase digestion. The ''M'' and ''B'' fractions were 0.5 ml; the ''T'' fraction was approximately 9 ml and is shown in ''C100'' after concentration to 0.5 ml in a Centricon microconcentrator. (B) YAC DNA was prepared in 100 μM spermine plus the NaCl concentrations indicated (67, 83 and 100 mM) and fractionated by centrifugation. The fraction sizes were smaller than in (A) ''T'' and ''M'' were 0.3 ml and ''B'' was 0.2 ml. ''MIB'' shows the YAC DNA in low salt microinjection buffer and ''SP'' shows the DNA in the presence of 100 μM spermine alone.

3.2. Polyamine-mediated DNA precipitation

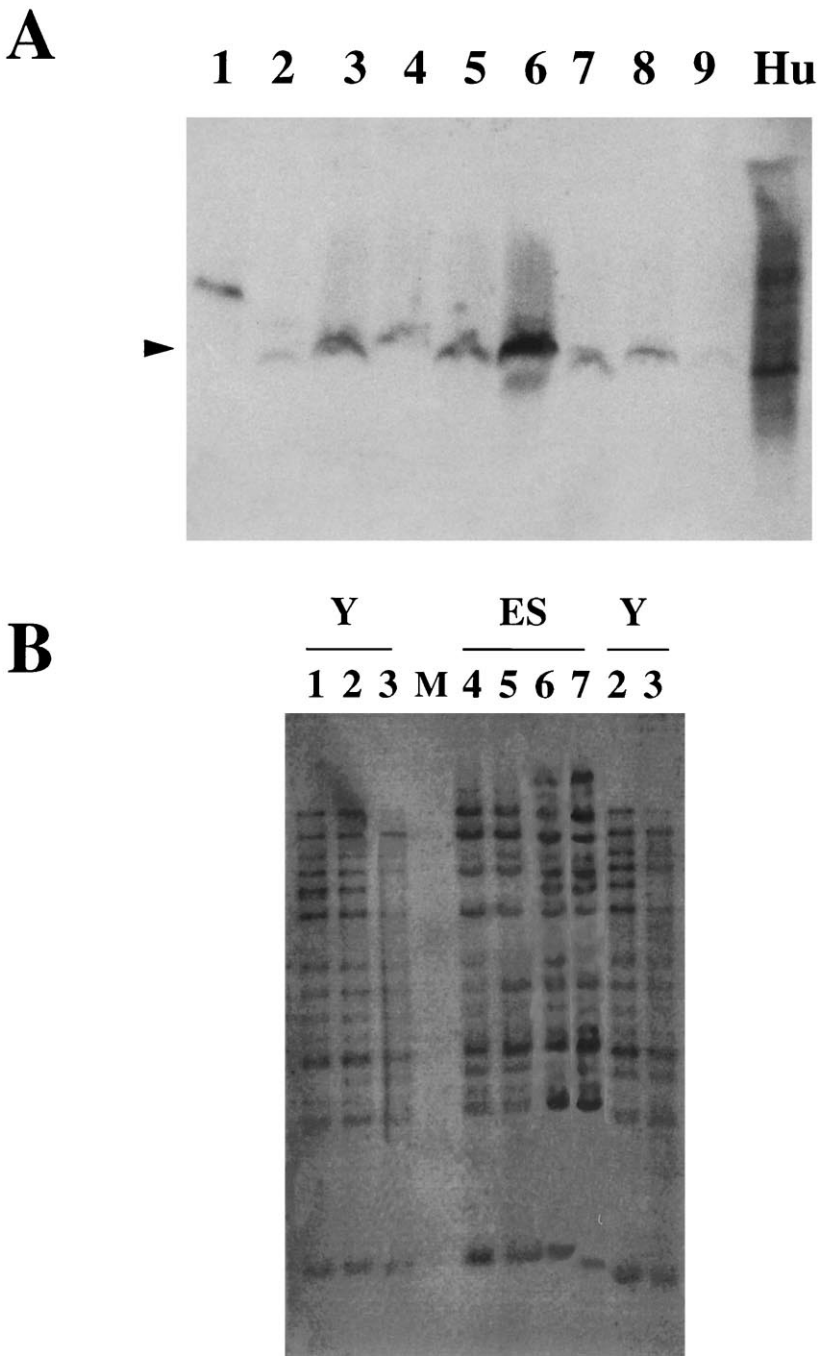
DNA precipitation can be a problem when using polyamine-compacted DNA [14,15]. In handling YAC DNA, we have found that concentrations above 4 ng/μl can occasionally lead to YAC DNA precipitation which can be hard to reverse. Despite the sharp transition between condensed and decondensed DNA as the sodium concentration is increased in the presence of a fixed concentration of polyamine [16], it is conceivable that an ideal concentration of sodium could be found which would reduce aggregation enough to avoid any risk of unwanted precipitation while maintaining a high degree of protection from breakage. However, our data indicate that while the presence of increased levels of sodium did prevent YAC aggregation and precipitation, it did so at the cost of protection from breakage. In Fig. 3, we compare the presence of 50 mM NaCl and 200 mM NaCl throughout the YAC isolation procedure. Aggregation was assessed by centrifugation, during which aggregated YACs pelleted while relatively monodisperse YACs remained in the supernatant; YAC breakage was monitored by CHEF electrophoresis. While the YACs remained soluble in the presence of 200 mM NaCl, they were broken during the purification. In 50 mM NaCl, the YACs remained unbroken but they aggregated and could be concentrated in the bottom fraction of a centrifuged YAC solution. A finer titration of sodium concentrations (Fig. 3(B)) confirmed that in 100 mM sodium, spermine-compacted YAC DNA does not precipitate but is broken (note the even distribution of YAC DNA smears in each fraction). In 67 mM sodium, the YACs were still precipitated but showed more breakage than at 50 mM. In 83 mM sodium, precipitation appeared to be reduced relative to that at 67 mM Na; however, in this experiment not all DNA precipitated even in the sample receiving spermine without any sodium, suggesting that the anti-aggregation effect of NaCl below 100 mM may not have been large. Often, approximately 50% of the total DNA remains in the upper fractions; this less aggregated DNA is not always seen by CHEF electrophoresis until it is concentrated (e.g. the C100 samples in Fig. 3(A)). Clearly, adding 100 mM Na along with 100 μM spermine is counterproductive when striving for maximum YAC protection. These data show that reducing the sodium concentration to 67 or 83 mM still permits DNA aggregation; thus, if there is an ''ideal'' sodium concentration that prevents aggregation while still protecting the YAC DNA from breakage, it must lie in a very narrow range of sodium concentrations between 83 and 100 mM.

3.3. YAC transfection

Based on the data described above, a protocol for YAC purification was devised which relied primarily on polyamines without any significant mono- or divalent cations in order to protect YAC DNA during isolation (Fig. 5). Table 1 shows a summary of ten YAC transfections into ES cells. In the first eight transfections listed, YAC DNA was isolated in 100 μM spermine. In some cases, NaCl was added as

Fig. 4. Pulsed-Field Gel and fingerprint assessment of YAC integrity. (A) Genomic DNA from ES cell lines transgenic for β-globin YACs (lanes 1–9) was prepared in agrarose plugs, digested with Sfil, run on a CHEF gel, blotted, and hybridized to a human ε-globin probe. The arrow indicates the positiion of a central, intact YAC band of 140 kb. Lane 1 shows a rearranged YAC, lane 6 a multicopy YAC with an additional broken fragment, and lane 9 and intact YAC of copy number less than one. The bands in lanes 3–5, 7, and 8 were deemed intact despite slight variations in mobility from gel to gel. Bands were quantitated relative to the corresponding human globin locus (''Hu'') and to single copy mouse genes (RET and Epo-receptor; not shown). (B) Genomic DNA from ES cells transgenic with β-globin YACs (''ES''; lanes 4–7) was digested with *Hin*dIII, electrophoresed, blotted, and probed with ^{32}P-labeled human ε-globin DNA. The resultant fingerprint bands were compared to the pre-transfected YAC DNA from yeast (''Y'', lanes 1–3). Three different YAC constructs are shown; ES DNAs shown in lanes 4 and 5 correspond to the Y DNA shown in the lanes numbered 3, ES lanes 6 and 7 correspond to Y lane 1, and the lanes numbered 2 correspond to a third construct. Untransfected murines ES cell DNA (''M'') was also run as a negative control. The YACs in lanes 4, 5 and 7 were intact as judged by CHEF analysis. The YAC in lane 6 showed two bands by CHEF analysis, one of which was the sized corresponding to an intact chromosome; the additional YAC fragment is not revealed by novel bands in the fingerprint.

indicated to the aggregated YAC DNA prior to transfection. In each experiment, we examined the transfection efficiency as well as the number of ES clones with intact YACs. Our data suggest that addition of 100–200 mM NaCl to the aggregated YAC DNA just prior to transfection may boost the transfection efficiency and thereby enhance the number of intact YACs recovered (Table 1, experiments 1–4). A titration of the NaCl added to the aggregated YAC DNA fraction indicates that an peak of intact YAC transfection may have occurred at 200 mM NaCl (Table 1, experiments 6–8).



Most YAC transfections discussed here were performed with a mixture of aggregated and nonaggregated YAC DNAs, since under the conditions employed, some aggregation of YAC DNA usually occurs; the presence of aggregated DNA becomes apparent because the YAC DNA is centrifuged to re-

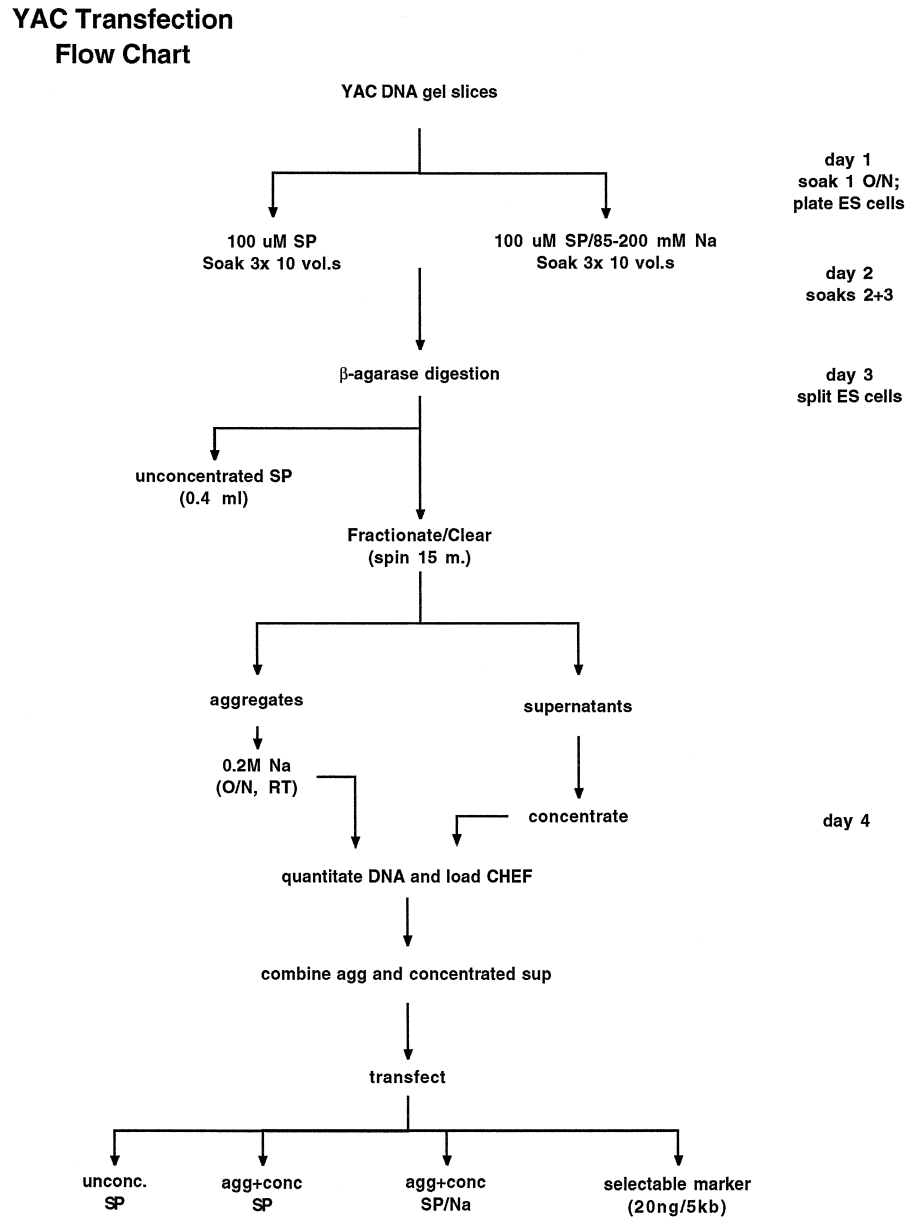
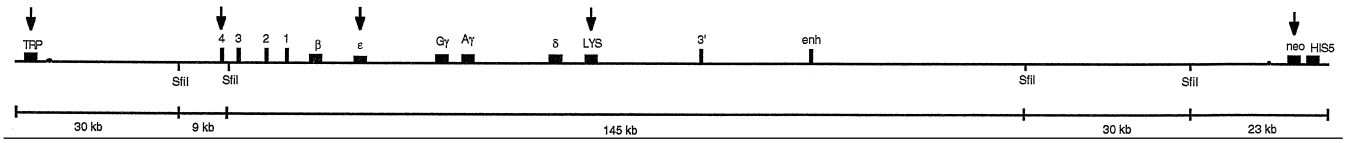


Fig. 5. Flow chart for YAC transfection. YAC DNA gel slices from a 250 ml CHEF gel were diced and stored at 4°C in CHEF gel running buffer. Four days before the transfection, the gel blocks were soaked in the buffers indicated (1/2 of the gel in 100 μM SP and optionally, 1/2 in 100 μM SP + 85–200 mM NaCl); ES cells were plated on a 10 cm dish at a density such thay they were confluent in two days. On third day, the gel blocks were digested with β-agarase. A 400 μl aliquot of the 100 μM SP sample was stored at 4°C for subsequent lipofection. The remaining YAC solutions were spun at 200 × *g* for 15 min to remove undigested agarose and fractionate larger YAC complexes from those less complexed. The aggregated YAC DNA (from 100 μM SP only – see text) was brought to 200 mM NaCl, and the ES cells were split to two 10 cm plates. On fourth day, the day of transfection, the YAC supernantants (sups) were concentrated and all the DNA fractions were quantitated; 1/10 volume of each was also loaded onto a CHEF gel. The aggregated (agg) and concentrated (conc) 100 μM SP fractions were combined and the samples indicated were lipofected into the ES cells.

Table 1
Summary of 10 YAC transfections



Expt.	Treatment	Final Na (mM)	C100 DNA (μg)	Aggreg. DNA (μg)	Total DNA (μg)	Colonies obtained	Neo$^+$ tested	LYS$^+$ hHS4$^+$ TRP$^+$	CHEF intact YACs	Without broken YACs
1	+ 100 Na	+ 45	0.84	1.3	2.2	28	27	15%	11%	7%
2	−	0	0.70	1.0	1.7	4	3	33%	33%	33%
3	+ 100 Na	+ 45	0.96	1.0	2.0	33	32	34%	31%	31%
4	−	0	0.74	1.3	2.1	3	3	33%	33%	33%
5	Unconc.	0	−	−	0.04	11	10	20%	20%	10%
6	+ 100 Na	+ 67	0.06	0.58	0.64	29	12	25%	25%	25%
7	+ 200 Na	+ 146	0.14	0.46	0.59	27	12	67%	67%	34%
8	+ 400 Na	+ 258	−	1.2	1.2	18	12	17%	1/1	0/1
9	200 Na/SP	200	1.1	−	1.1	20	16	31%	19%	13%
10	5 mM SP	0	−	−	0.24	2	2	−	1/1	1/1

β-globin YACs were transfected into CCE embryonic stem cells as described in Section 2. Experiments 1–8 represent two groups of four transfections performed with YAC DNA isolated in the presence of 100 μM spermine (SP); each group of four transfections was performed in parallel. In the ''200 Na/SP'' sample of experiment 9, 100 μM spermine and 200 mM NaCl were present throughot the YAC purification; this YAC DNA is shown in Fig. 3(A). The ''5 mM SP'' YAC DNA of experiment 10 was isolated in 5 mM spermine. ''+ 100 Na'' indicates that NaCl was added to a final concentration of 100 mM to the aggregated DNA fraction (''aggreg''). ''Final Na'' refers to the Na concentration in the combined aggregate and concentrated supernatuant (''C100'') fractions just prior to lipofection. The volumes of total DNA transfected ranged from 310–530 μl. Colonies are the number of neomycin resistant colonies obtained from a transfection. Percentages are based on a subset of colonies positive for the neomycin gene by PCR (''neo$^+$'') which were further analyzed. LYS$^+$, hHS4$^+$, and TRP$^+$ refer to colonies positive by PCR at the corresponding sites (arrows) on the map of a representive YAC (β^{up}) shown below the Table (to scale; the hypersentitive sites are labeled 1–4 at the 5′ end of the globin locus and ''3'' at the 3′ end of the locus). The percentage of intact YACs were determined by CHEF analysis. The number of ES lines without an additional broken YAC are indicated; in most cases (two-thirds of those listed in the ''without broken'' column), intact YACs wer present at 1–2 copies/cell. Approximately 5% of transfectants had a YAC copy number less than 1. The level of unconcentrated DNA (''C100'') in the ''+ 400 Na'' sample was undetectable.

move any undigested agarose and to concentrate the DNA in microconcentrators. We show here that aggregated DNA alone is able to transfect ES cells (for example in experiment 8 of Table 1, as well as in other experiments not shown). It is also clear from our data that the YAC DNA does not need to be concentrated in order to obtain useful YAC transfectants (see experiment 5), although we find that the use of unconcentrated YAC DNA is generally less efficient in producing as large a number of transfectants or as high a percentage without broken YACs as using concentrated YAC DNA (for example compare experiments 3, 6, and 7). Nonetheless, we believe that it can be useful to transfect an aliquot of unconcentrated YAC DNA as a hedge against unexpected precipitation due to high DNA levels. Finally, although the reduction of the spermine concentration from 5 mM [17] to 100 μM may have reduced the incidence of spontaneous DNA precipitation [18], we also show that it is possible to transfect ES cells with YACs condensed in 5 mM spermine (experiment 10, Table 1).

The data in Table 1 also show that using PCR to scan the genomic DNA of ES cell transfectants for markers present throughout the YAC, including the arm distal to the selectable marker, provides an excellent indicator of whether at least one intact YAC is present in the transfectant (as judged by CHEF gel analysis and Alu fingerprinting; Fig. 4). Fingerprinting alone, however, may not be sufficient to demon-

strate intactness; up to 50% of ES cells containing YACs with complete Alu fingerprints actually contain an intact YAC along with a broken one (Table 1, experiments 5 and 7 and Fig. 4(A)), and it can be difficult to discriminate the alteration in fingerprint band intensities or novel bands produced by the presence of a truncated but otherwise unrearranged YAC (Fig. 4(B), lane 6).

The presence of both 200 mM NaCl and 100 μM spermine throughout the YAC purification (experiment 9, Table 1) shows that, despite the substantial breakage of DNA (Fig. 3(A)), intact YAC transfectants could be obtained at a useful frequency (Table 1). Therefore, it is feasible to transfect YAC DNA prepared in this manner to further mitigate the risk of YAC precipitation beyond useful levels (Fig. 5), although we have found that such a procedure is almost never necessary.

3.4. Variability and further modifications: Effects of fetal bovine serum and lipofection reagent on transfection efficiency

Occasionally, a reduced number of transfectants was produced when concentrated YAC DNA was used for transfection, even when the DNA was intact and of sufficient quantity just prior to lipofection. By following control transfections of a 5 kb PGKneo selectable marker DNA, it was determined that the efficiency of the lipofection was an important variable in YAC transfections. Using 20 ng of a 5 kb gel-purified PGKneo fragment as a positive control for transfection, we found two influences on the efficiency of lipofection. First, variations between lots of lipofection reagent (DOTAP) result in a several-fold difference in transfection efficiency. With the better lots of lipofection reagent, twenty nanograms of PGKneo produced at least 35–45 transfectants; (the transfection efficiency for a given lot of DOTAP is available from the manufacturer upon request). Second, the presence of residual fetal bovine serum during the initial exposure of ES cells to the lipid/DNA complexes had a very substantial inhibitory effect on the transfection efficiency (Table 2), despite claims that the presence of sera does not affect lipofection with this reagent [8]. Thus, we found that extensive washing of the cells before lipofection had a significant effect on transfection efficiency. Although the data in Table 2 also suggest that LIF may have had a beneficial effect on transfection efficiency, it was observed that when larger amounts of LIF in the form of a CHO cell supernatant was present immediately after transfection, the transfection efficiency of YAC DNA was noticeably reduced; purified LIF (ESGRO; GIBCO-BRL) showed no negative effect on transfection (John Yang, personal communication).

Table 2
Fetal Bovine Serum Inhibits Transfection

	FBS conc.	Colonies	Ratio
FBS^- medium	0.06%	2	1×
+ 1× wash	0.0006%	25	12.5×
+ 2× wash	0.000006%	40	20×
+ 2 + LIF O/N	0.000006%	54	26×
+ 2 + FBS O/N	0.000006%	33	16.5×
FBS^+ medium	15%	0	< 0.5×

ES cells (1.6×10^7 one day after being split 1:2) were lipofected with 20 ng of a 5 kb gel-purified fragment containing a neomycin gene driven by a PGK promoter (PGKneo). ''FBS^-'' and ''FBS^+'' indicate the media in which the cells were resuspended prior to exposure to the DNA-lipid complexes; ''FBS^+'' is ES cell medium (Section 2). Cells and the recipient plate were washed as indicated in 10 ml FBS^- medium or PBS. ''LIF O/N'' and ''FBS O/N'' refer to a LIF-rich CHO cell supernatant added to a concentration of 0.04% and fetal bovine serum (to 15%), respectively, which were added to 2× washed tissue culure plates immediately after transfection. All media were changed to ES (15% FBS) 12 h after lipofection. G418 resistant colonies (200 μg/ml) were selected beginning 2 days after the transfection; colonies were counted after 13 days of selection. ''Ratio'' indicates the number of colonies obtained relative to the FBS- transfection (0.06% FBS present during lipofection).

The choice of lipofection reagent may also make a difference, but this was less clear. In one side-by-side test, the efficiency of a non-hydrolyzable lipofection reagent (Lipofectin, GIBCO-BRL) was approximately 5-fold lower than the reagent containing DOTAP. However, others have used the Lipofectin reagent to produce YAC transfected DNA [2], so it is possible that lot differences accounted for our results. A third method which we found to enhance the lipofection of YACs into ES cells was to add sodium chloride to the aggregated YAC DNA. As mentioned above, our data suggested that the percentage of large, intact YACs recovered was maximized with the addition of approximately 200 mM NaCl to the aggregated YAC DNA (Table 1).

4. Discussion

4.1. Comparison of protocols

The first publication showing protection of YACs from breakage employed a high concentration of spermine (5 mM [17]). It was subsequently shown that spermine-protected YACs could be used to transfect fibroblasts [19]. This procedure used a greatly reduced spermine concentration (50 μM) in order to avoid DNA precipitation, and in addition, adherent cells were lipofected in situ. We modified the fibroblast procedure, which we found did not work adequately for ES cells. Our protocol for ES cell transfection by YACs was similar to a modified technique published by Strauss and Jaenisch [20]. In both procedures, YAC DNA was compacted in spermine, ES cells were transfected in suspension, and a cellularly hydrolyzable and therefore less bioactive or toxic lipofection reagent containing DOTAP [21,22] was used. Among the differences, we found that YAC concentration could be useful and we did not find the use of poly-L-lysine, another polycationic condensing agent, to be necessary for YAC protection. The net yield of transfected ES colonies from these two spermine-based protocols was very similar; however, the percentage of intact YACs obtained appears to be considerably higher in our protocol (as high as 67% with the average being 35%; Table 1) compared to that reported by Strauss et al. (7%).

Other approaches to YAC transfection of ES cells have been taken. In one, YAC DNA was not modified to carry a selectable marker and was not protected by compaction before being released from low melting point agarose. Instead, the YAC DNA was mixed with a selectable marker and carrier DNA and then transfected without further concentration by using a cationic lipid (Transfectam, Promega; [23]). In the best case of this approach published [24], 2–3 intact YACs were obtained from an initial screen of 240 G418 resistant transformants (approximately 1% intact). In this approach, far more colonies must be picked for analysis than in the spermine-based protocols compared above and the recovery of intact YACs is not high. In addition, no evidence was given for compaction of the YACs by the cationic lipid; our data suggest that the monovalent cations in the ES cell medium will probably decompact spermine-compacted YACs. In another variant of spermine based transfection, higher concentrations of spermine (500 μM–1 mM) have been used successfully to produce two colonies with an intact YAC out of 23 transfected (9%) [2]. This is consistent with our observation that even spermine concentrations up to 5 mM can be employed for YAC transfections. Finally, as indicated earlier, YACs have been introduced into several types of cells in vitro by fusion to yeast spheroplasts. Introduction of a 670 kb human HPRT YAC into ES cells by fusion [7] produced 8 ES transfectants with an intact, single copy YACs out of 20 total transfectants (40%), a frequency higher than obtained with most spermine-mediated transfer protocols. Despite integration of large amounts of the yeast genome, the authors were able to obtain germline transmission. For applications in which preventing introduction of yeast DNA into the target cells is not an issue, fusion appears to be a simple and effective method by which low copy number YACs can be introduced into cells.

One difficulty in comparing different published protocols is that in many cases very limited, if any, evidence of the intactness of YAC transgenes is given. Relying on gene expression as an assay for intactness, for example, can be misleading if the gene of interest does not span the center of the YAC where shear forces are most likely to produce breakage of uncompacted DNA [12]. Furthermore, inter-Alu fingerprinting alone is unlikely to be sufficient to show intactness of a YAC integrant [3], since a relatively small amount of the YAC DNA is scanned by the PCR. Even with fingerprinting based on Southern blots, it can be difficult to discriminate the alteration in fingerprint band intensities produced by the presence of a truncated but otherwise unrearranged YAC (Section 3); furthermore, the junctions at the site of an intact YAC integration can produce two bands unique to each fingerprint. Therefore, it is recommended that pulsed-field gel analysis always be performed to assess the general integrity of the YAC integrations by hybridization to probes to the central portion of the YAC most likely to break in noncompacted DNA, as well as to the region spanning the locus of interest. Then, expression should be analyzed in several transfected clones carrying intact YACs in order to account for the effect of more subtle mutations which are not evident by pulsed-field

gel and fingerprinting. In examining the expression of genes contained within a YAC insert, it should be noted that sequences neighboring the YAC, such as endogenous sequences at the site of integration or any cotransfected yeast DNA, might cause position effects which would lead to variable expression from genes in the YAC insert despite intact integration of the YAC. Thus, valid expression studies using YACs may require the investigator to determine that the construct of interest contains the appropriate elements which provide copy-dependent and position-independent expression [25].

4.2. Precipitation used to advantage

Although precipitation of polyamine condensed YACs can lead to loss of DNA, YAC condensation can be used to some advantage, not only for optimal protection from DNA breakage, but also as a convenient concentration step. It has long been known that polyamines can precipitate DNA [26]. Nonetheless, monodisperse polyamine-condensed YACs are soluble in aqueous solutions. They do, however, aggregate due in part to crosslinking by the polyamines [27], a process which is more pronounced at higher temperatures [16]. In our studies, the degree of precipitation was dependent on both DNA concentration and total centrifugal force applied (as is generally true for DNA precipitations), as well as on the time between polyamine exposure and transfection of the DNA. Under our conditions, usually approximately 50% (but up to 100%) of the DNA was ''precipitated'', i.e. loosely concentrated by centrifugation; this material could be used for transfections. However, if pelleting was carried too far, e.g. a true, solid pellet was formed, the YACs became very difficult to resuspend, even in salts up to 0.4 M Na. This may be a function of extensive entanglements occurring in the pellet. In contrast, small polyamine-compacted DNAs can be readily resuspended from a tight pellet in high salt buffers [14]. We have attempted to decompact the mildly aggregated YACs more rapidly by increasing the Na concentration in small increments over short periods of time with vigorous pipetting as is done in some gene therapy protocols employing poly-L-lysine [28]. Under these conditions, however, the YAC DNA was broken. Despite the transfectability of aggregated YAC DNA shown here, it is nonetheless possible that the YAC transfection efficiency could be further enhanced by relying solely on the more monodisperse YACs left behind in the supernatant after aggregated YACs are spun out, e.g. by doubling the amount of concentrated supernatant used.

Several groups have attempted to increase the amount of YAC DNA available for transfection. In some experiments, YACs were modified to include a conditional active centromere and a selectable marker so that the YAC DNA could be amplified in the yeast relative to the endogenous chromosomes [12,29,30]. Our studies with ES cell transfection show that use of amplifiable YACs is definitely not necessary for YACs of 250 kb. In addition, we observed reduced recoveries of YACs from cells exposed to amplification conditions, as well as YAC rearrangements (unpublished data). We found that with an effective lot of the transfection lipid, transfectants with intact YACs could be obtained from very small quantities of YAC DNA, e.g. 40 ng of 250 kb DNA (e.g. Table 1, experiment 5); this represents a much higher ratio of transfectants to input DNA molecules than we have obtained with the 5 kb marker DNA, PGKneo, suggesting that the larger DNAs may be more transfectable. We also found that use of pulsed field DNA plugs with a yeast concentration greater than 6×10^9 per ml led to a significant loss of resolution of the YAC DNA. Furthermore, the use of plugs with a final yeast concentration greater than 5×10^9 per ml may correlate with a greater risk of YAC aggregation in the agarose slices during exposure to polyamines. Thus, further reductions in the starting concentration of yeast DNA, and therefore of the DNA exposed to polyamines, might reduce the risk of precipitation.

4.3. YAC protection: the mono- vs. polycation controversy

The experiments presented here show that among the general approaches used to produce intact, purified YACs for lipofection into ES cells, the reliance on NaCl for protection from DNA breakage is not ideal. The effect of sodium on DNA compaction is a general phenomenon and therefore the same considerations should apply for microinjection of YAC DNA. This view is supported by previously published experiments using methods different from those em-

ployed here. For example, it has been shown that increasing the Na concentration can allow some reduction in the radius of gyration of DNAs by reducing the stiffness of the DNA [31]. In going from 5 to 100 mM NaCl the radius of gyration of DNA drops about 20%. Even less change is seen by increasing the salt concentration further; the radius of gyration at 4 M is 38% reduced from the 5 mM value [31]; in 6 M monovalent salts, DNA still does not condense into the compact torus seen in the presence of cations of valence three of greater [18,27,32]. Consistent with our results showing that 100 mM NaCl is not very protective of YAC integrity, Peterson et al. used a 100 mM NaCl-based protocol to microinject a 250 kb β-globin YAC into mice and noted that it was quite difficult to obtain intact YAC integrations [33]. Since most of the YACs they describe appeared to be broken near the center of the YAC, our interpretation is that the YACs may have been snapped in two during manipulation, rather than having been broken through cellular processes.

Some YAC microinjection protocols have employed a mixture of polyamines and NaCl in an effort to maintain the protective effect of polyamine condensation of DNA while reducing the risk of precipitation [12]. EM data from a recent study [15] showed that the diameter of 250–1000 kb YACs ''tightly gathered'' in 100 μM polyamines/100 mM NaCl was roughly 2 μm, which is very close to the size predicted by Gnirke et al. for a 500 kb YAC in 100 mM NaCl alone. This is consistent with an antagonistic effect of mono- and divalent cations on polyamine-mediated condensation [18,34]. Although one might expect to get some shearing of YACs with a 2 μm radius of gyration when passing them through microinjection needles which are approximately 0.5–1.0 μm in diameter at the tip (by comparison polyamine condensed YACs are 0.05–0.1 μm [18,35,36]), our data show that when YACs in 100 mM NaCl or 100 mM NaCl/100 μM polyamines are passed through 12 μm diameter needles or even relatively wide 200 μl pipet tips, they can be easily broken. Therefore, in addition to reducing the radius of gyration, polyamine condensation is probably providing some structural stability, such as from intramolecular crosslinking, that is lacking in the NaCl treated samples. Although polyamine aggregated YACs might not pass through standard microinjection needles without blockage or breakage, the relatively monodisperse YAC fraction remaining after centrifugation should do so without damage. The value, if any, of adding sodium to the polyamine buffer during purification is not that it provides optimal protection (Figs. 1 and 2), but rather that it might reduce the risk of an irreversible precipitation of aggregated YAC DNA (Fig. 3). In spite of the presence of a high percentage of broken YACs, DNA isolated in 100 μM spermine and 200 mM Na (Fig. 3(A)) generated a percentage of intact YACs similar to some of the spermine-only transfections (Table 1).

Although the protective benefit of polyamines is clear, our CHEF data also show that in the absence of polyamines, and even in the absence of 100 mM NaCl, intact YACs can survive manipulation. Therefore, the gentler the YAC purification procedure, the greater the yield of intact YACs will be regardless of the buffer employed. This is the likely explanation for the ability of YACs isolated in relatively nonprotective salts to produce transgenic mice.

In summary, our data show that compaction, precipitation, and breakage are linked; as the YACs are decompacted they precipitate less and break more. This most likely means that the sodium ions are not preferentially disrupting intermolecular polyamine linkages in favor of intramolecular ones. As intermolecular linkages decrease, the YACs are less susceptible to precipitation; as intramolecular linkages decrease, the YACs are more susceptible to breakage. We base the protocol presented here on the view that it is best to purify and transfect YACs in the most highly protected state, i.e. with polyamines in low sodium buffer. We believe that the procedure described increases the number of useful YACs obtained, meaning those which are low copy, intact, and without additional broken fragments. These conditions may also be useful for preparation of YAC DNAs for microinjection, and may improve the frequency of intact YAC transgenic mice.

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References

[1] G.H. Nonet, G.M. Wahl, Introduction of YACs containing a putative mammalian replication origin into mammalian cells can generate structures that replicate autonomously, Somat. Cell. Mol. Genet. 19 (1993) 171–192.
[2] B.T. Lamb, S.S. Sisodia, A.M. Lawler, H.H. Slunt, C.A. Kitt, W.G. Kearns, P.L. Pearson, D.L. Price, J.D. Gearhart, Introduction and expression of the 400 kilobase precursor amyloid protein gene in transgenic mice, Nature Genet. 5 (1993) 22–29.
[3] D.J. Smith, Y. Zhu, J. Zhang, J. Cheng, E.M. Rubin, Construction of a panel of transgenic mice containing a contiguous 2-Mb set of YAC/P1 clones from human chromosome 21q22.2, Genomics 27 (1995) 425–434.
[4] V. Pachnis, L. Pevny, R. Rothstein, F. Costantini, Transfer of a yeast artificial chromosome carrying huma DNA from *Sacchaomyces cerevisiae* into mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 5109–5113.
[5] W.J. Pavan, P. Hieter, R. Reeves, Modification and transfer into an embryonal carcinoma cell line of a 360-Kilobase human-derived yeast artficial chromosome, Mol. Cell. Biol. 10 (1990) 4163–4169.
[6] C. Huxley, Y. Hagino, D. Schlessinger, M.V. Olson, The human HPRT gene on a yeast artificial chromosome is functional when transferred to mouse cells by cell fusion, Genomics 9 (1991) 742–750.
[7] A. Jakobovits, A.L. Moore, L.L. Green, G.J. Vergara, C.E. Maynard-Currie, H.A. Austin, S. Klapholz, Germ-line transmission and expression of a human-derived yeast artificial chromosome, Nature 362 (1993) 255–258.
[8] Boehringer Mannheim, Transfection reagent (DOTAP), 1993.
[9] J.M. Ure, S. Fiering, A.G. Smith, A rapid and efficient method for freezing and recovering clones of embryonic stem cells, Trends Genet. 8 (1992) 6.
[10] W.J. Pavan, P. Hieter, R.H. Reeves, Generation of deletion derivatives by targeted transformation of human-derived yeast artificial chromosomes, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1300–1304.
[11] G. Church, W. Gilbert, Genomic sequencing, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 1991–1995.
[12] A. Schedl, L. Montoliu, G. Kelsey, G. Schutz, A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice, Nature 362 (1993) 258–261.
[13] A. Gnirke, C. Huxley, K. Peterson, M.V. Olson, Microinjection of intact 200-500 kb fragements of YAC DNA into mammalian cells, Genomics 15 (1993) 659–667.
[14] B.C. Hoopes, W.R. McClure, Studies on the selectivity of DNA precipitation by spermine, Nucleic Acids Res. 9 (1981) 5493–5504.
[15] L. Montoliu, C.T. Bock, G. Schutz, H. Zentgraf, Visualization of large DNA molecules by electron microscopy with polyamines: application to the analysis of yeast endogenous and artificial chromosomes, J. Mol. Biol. 246 (1995) 486–492.
[16] J. Widom, R.L. Baldwin, Cation-induced toroidal condensation of DNA: studies with $Co^{3+}(NH_3)_6$, J. Mol. Biol. 144 (1980) 431–453.
[17] L.B. Couto, E.A. Spangler, E.M. Rubin, A method for the preparative isolation and concentration of intact yeast artificial chromosomes, Nucleic Acids Res. 17 (1989) 8010.
[18] D.K. Chattoraj, L.C. Gosule, J.A. Schellman, DNA condensation with polyamines. II. Electron microscopic studies, J. Mol. Biol. 121 (1978) 327–337.
[19] W.M. Strauss, R. Jaenisch, Molecular complementation of a collagen mutation in mammalian cells using yeast artificial chromosomes, EMBO J. 11 (1992) 417–422.
[20] W.M. Strauss, J. Dausman, C. Beard, C. Johnson, J.B. Lawrence, R. Jaenisch, Germ line transmission of a yeast artificial chromosome spanning the murine α_1(I) collagen locus, Science 259 (1993) 1904–1907.
[21] L. Stamatatos, R. Leventis, M.J. Zuckermann, J.R. Silvius, Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes, Biochemistry 27 (1988) 3917–3925.
[22] R. Levenits, J.R. Silvius, Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles, Biochim. Biophys. Acta 1023 (1990) 124–132.
[23] T.K. Choi, P.W. Hollenbach, B.E. Pearson, R.M. Ueda, G.N. Weddell, C.G. Kurahara, C.S. Woodhouse, R.M. Kay, J.F. Loring, Transgenic mice containing a human heavy chain immunoglobulin gene fragment cloned in a yeast artificial chromosome, Nature Genet. 4 (1993) 117–123.
[24] B.E. Pearson, T.K. Choi, Expression of the human β-amyloid precursor protein gen from a yeast artifcial chromosome in transgenic mice, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10578–10582.
[25] D.I. Martin, S. Fiering, M. Groudine, Regulation of β-globin gene expression: straightening out the locus, Curr. Opin. Genet. Dev. 6 (1996) 488–495.
[26] S. Razin, R. Rozansky, Mechanism of the antibacterial action of spermine, Archiv. Biochem. Biophys. 81 (1959) 36–54.
[27] S.A. Allison, J.C. Herr, J.M. Schurr, Structure of viral phi29 DNA condensed by simple triamines: a light-scattering and electron-microscopy study, Biopolymers 20 (1981) 469–488.
[28] J.C. Perales, T. Ferokol, M. Molas, R.W. Hanson, An evaluation of receptor-mediated gene transfer using DNA-ligand complexes, Eur. J. Biochem. 226 (1994) 255–266.
[29] A. Schedl, F. Beermann, E. Thies, L. Montoliu, G. Kelsey, G. Schutz, Transgenic mice generated by pronuclear injection of a yeast artificial chromosome, Nucleic Acids Res. 20 (1992) 3073–3077.

[30] L. Montoliu, A. Schedl, G. Kelsey, P. Lichter, Z. Larin, H. Lehrach, G. Schutz, Generation of transgenic mice with yeast artificial chromosomes, Cold Spring Harbor Symp. Quant. Biol. LVIII (1993) 55–62

[31] N. Borochov, H. Eisenberg, Z. Kam, Dependence of DNA conformation on the concentration of salt, Biopolymers 20 (1981) 231–235.

[32] I. Baez, P. Gargiglio, L.M. Rangel, P. Chavez, L. Cervantes, C. Arguello, C. Wong, C. Montanez, Electron microscopy and biochemical properties of polyamine-compacted DNA, Biochemistry 26 (1987) 6387–6392.

[33] K.R. Peterson, Q.L. Li, C.H. Clegg, T. Furukawa, P.A. Navas, E.J. Norton, T. Kimbrough, G. Stamatoyanopoulos, Use of yeast artificial chromosomes (YACs) in studies of mammalian development: production of β-globin locus YAC mice carrying human globin developmental mutants, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 5655–5659.

[34] D. Kaiser, H. Tabor, C.W. Tabor, Spermine protection of coliphage λ DNA against breakage by hydrodynamic shear, J. Mol. Biol. 6 (1963) 141–147.

[35] L.C. Gosule, J.A. Schellman, Compact form of DNA induced by spermidine, Nature 259 (1976) 333–335.

[36] K.A. Marx, G.C. Ruben, Studies of DNA organization in hydrated spermidine-condensed DNA toruses and spermidine-DNA fibres, J. Biomol. Struct. Dyn. 1 (1884) 1109–1132.